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Decolorization and Detoxification of Extraction-Stage Effluent from Chlorine Bleaching of Kraft Pulp by *Rhizopus oryzae*

R. NAGARATHNAMMA[1] AND PRATIMA BAJPAI[2]*

School of Biotechnology, Thapar Institute of Engineering & Technology,[1] and Chemical Engineering Division, Thapar Corporate Research & Development Centre,[2] Patiala 147 001, India

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***Rhizopus oryzae*, a zygomycete, was found to decolorize, dechlorinate, and detoxify bleach plant effluent at lower cosubstrate concentrations than the basidiomycetes previously investigated. With glucose at 1 g/liter, this fungus removed 92 to 95% of the color, 50% of the chemical oxygen demand, 72% of the adsorbable organic halide, and 37% of the extractable organic halide in 24 h at temperatures of 25 to 45°C and a pH of 3 to 5. Even without added cosubstrate the fungus removed up to 78% of the color. Monomeric chlorinated aromatic compounds were removed almost completely, and toxicity to zebra fish was eliminated. The fungal mycelium could be immobilized in polyurethane foam and used repeatedly to treat batches of effluent. The residue after treatment was not further improved by exposure to fresh *R. oryzae* mycelium.**

Conventional pulp bleaching uses a variety of chlorine species as bleaching agents. The advantage of using chlorine is simply that it is cheap and effective. Bleaching with chlorine chemicals usually starts with an acid treatment with elemental chlorine at low temperature, pH, and consistency. During chlorination, wood components—lignin and some carbohydrates—are structurally modified, degraded, and chlorinated. Some of these chlorinated compounds (mostly low-molecular-weight material) are dissolved into the spent chlorination liquor. This stage is followed by an alkaline extraction stage using high temperature, pH, and consistency. In the extraction stage, chlorinated, oxidized lignins, not soluble in the acidic chlorination stage, are solubilized and dissolved into the spent liquor. The final bleaching is achieved by using oxidizing chemicals, usually chlorine dioxide and hydrogen peroxide. The substances being dissolved in the latter stages are more strongly oxidized, and pollution loads from these stages are minor. The pulp mills in Scandinavia and North America have stopped using chlorine bleaching so that they can meet government regulations on adsorbable organic halide (AOX) discharge. In India, bleaching is still being done with chlorine. Chlorine dioxide is used by very few mills for viscosity protection in the first bleaching stage (10 to 15% substitution) and for brightening in the final bleaching stages. Oxidative extraction and peroxide bleaching are also being tried in some mills.

Most mills are reluctant to recycle bleach plant effluent to the chemical recovery system due to the corrosive nature of chloride ion and the substantial dilution of the chemicals to be recycled. Chlorinated organics generated during pulp bleaching not only exert an oxygen demand (biochemical oxygen demand [BOD] and chemical oxygen demand [COD]) but also cause effluent color and toxicity (acute and chronic) (4, 5, 22, 26, 40). Chlorinated organics in spent bleaching liquor are also responsible for the mutagenicity of the effluent (34). The low-molecular-weight fraction of the chlorolignins is the main contributor to the effluent BOD and acute toxicity. The high-molecular-weight chlorinated compounds contribute little to BOD and acute toxicity, due to their inability to pass through cell membranes. They are the major contributor to effluent color, COD, and chronic toxicity (17, 22). Color not only is aesthetically unacceptable but also inhibits the natural process of photosynthesis in streams due to absorbance of sunlight (22). This leads to chains of adverse effects on the aquatic ecosystem, as the growth of primary consumers as well as secondary and tertiary consumers is adversely affected. Discharge of untreated or partially treated wastewaters from pulp and paper mills results in persistence of color in the receiving body over a long distance. Under natural conditions, these compounds are slowly degraded to various chlorinated phenolics which may be methylated under aerobic conditions (17). The low-molecular-weight phenolics and their methylated counterparts (which are more lipophilic) cause toxicity and are bioaccumulable in fish (17). However, Archibald et al. (3) have reported that toxic levels of low-molecular-weight chlorinated compounds do not accumulate during the natural degradation of chlorolignins. They found that the effluents indeed appear to stimulate the growth of algae and primary consumers, probably because of the nutrients they contain. Work by Millar and Carey (31) has shown that chlorolignins in biotreated effluents disappear with half-lives of a few weeks as a result of both photolysis and biodegradation. O'Connor and Voss (33) showed that the release of monomeric chlorinated phenolic compounds from chlorolignin preparations during storage was caused by slow desorption of sorbed compounds and was limited to 2% of the amount of chlorinated phenolic compounds in the extraction-stage effluent.

About 75% of the dissolved organic material, 60% of the COD load, 40 to 50% of the organically bound chlorines, and 80% of the color-imparting substances of bleach plant effluents are reportedly contributed by extraction-stage effluents. Therefore, a treatment method that can degrade, dechlorinate, and decolorize extraction-stage effluent can tackle most of the environmental pollution problems associated with bleach plant effluents (4, 5).

Among the biological methods tried so far, methods using wood-degrading white rot fungi have been reported to have the potential to successfully treat these effluents (4, 5). The enzyme system of the white rot fungi includes a group of nonspecific extracellular enzymes which catalyze not only degradation of lignin and chlorolignins but also oxidation of several persistent aromatic and halogenated compounds like lindane,

* Corresponding author. Mailing address: Chemical Engineering Division, Thapar Corporate Research and Development Centre, Patiala 147 001, India. Phone: 0175-393566. Fax: 0175-212002. E-mail: pratima@tcrdcpt.ren.nic.in.

TABLE 1. Characteristics of the effluent used

Parameter	Value for alkaline extraction-stage effluent[a]
Color (PCU)	7266 ± 251.6
COD (mg/liter)	1359 ± 52.5
BOD (mg/liter)	418 ± 20.2
AOX (mg/liter)	58.6 ± 1.52
EOX (mg/liter)	1.33 ± 0.11
pH	10.2 ± 0.25
Alkalinity (mg/liter as $CaCO_3$)	726 ± 64.2
Total solids (mg/liter)	3340 ± 50
Dissolved solids (mg/liter)	3000 ± 00
Suspended solids (mg/liter)	253 ± 50.3

[a] Results are reported as means of three measurements ± the standard deviation.

DDT, PCP, benzopyrene, creosote, coal tars, and heavy fuels, etc. (8, 9, 16). The serious drawback associated with use of fungi for decolorization of bleach plant effluents has been the requirement for an easily metabolizable cosubstrate like glucose for the growth and development of ligninolytic activity. Use of cosubstrate results in increased cost of treatment and even net increase in COD in the effluents.

While fungal strains were being screened for the requirement for less or no cosubstrate during decolorization, a member of the *Zygomycetes*, *Rhizopus oryzae*, was found to efficiently decolorize and dechlorinate bleach plant effluent at a relatively low cosubstrate concentration. This paper investigates decolorization and dechlorination of bleach plant effluent, especially extraction-stage effluent, by *R. oryzae*.

MATERIALS AND METHODS

Effluent source. Effluent from the first alkaline extraction stage was used since it is a major source of color in bleach plants. Extraction-stage effluent was obtained from a large paper mill utilizing eucalyptus as the main raw material. Starting with a kappa number of 20, the mill produces pulp of 86 to 87% ISO brightness by using a CEDD bleaching sequence (where C is chlorination, E is alkaline extraction, and D is chlorine dioxide) and a chlorine multiple of about 0.22. The effluent was neutralized with sulfuric acid, filtered through a 0.5-mm-pore-size sieve to remove large suspended particles, and stored at 4°C.

Cultures. More than 110 fungal strains were isolated from natural sources. Cultures were grown in potato dextrose broth. Stock cultures of the fungi were stored on potato dextrose slants at 4°C and periodically subcultured.

Culture conditions. For screening of fungi requiring less cosubstrate, medium containing 1 g of glucose per liter was used. In addition to glucose, the medium contained 1.5 g of calcium chloride, 2.0 g of magnesium sulfate, 1.5 g of potassium dihydrogen phosphate, and 0.15 g of ammonium chloride in 1 liter of extraction-stage effluent (pH 4.5). The medium was sterilized by autoclaving. Inoculation was carried out with mycelia precultured in potato dextrose medium (pH 5.5). Cultures were incubated in 250-ml Erlenmeyer flasks with 50 ml of medium on a rotary shaker (200 rpm) at 30 ± 1°C. Color removal was measured for up to 3 days, with a sample being taken every day.

The kinetics of decolorization and dechlorination was studied by using optimized medium and conditions in batch culture. Samples were analyzed for color, COD, AOX, and extractable organic halide (EOX).

Decolorization and COD reduction were also studied with immobilized fungus in a repeated batch process. The fungus was immobilized on polyurethane foam (29). The foam was cut into 1-cm^3 cubes, washed three times with distilled water, and sterilized at 121°C for 15 min. Afterwards, the sterilized cubes (1 g) were transferred to 250-ml Erlenmeyer flasks containing 25 ml of potato dextrose broth. The flasks were inoculated with the fungus and incubated at 30 to 32°C with shaking at 200 rpm for 7 days. The foam-immobilized mycelium was used repeatedly for decolorizing the extraction-stage effluent for 12 days. The incubation liquor was decanted and replaced with fresh effluent every 24 h. Color and COD reduction were monitored daily for 12 days. A few samples were also analyzed for AOX.

Analytical methods. COD was determined by the closed reflux colorimetric method (standard method 5220-D, 1989) (14). An HACH COD reactor was used for digestion of the sample in COD vials. COD was spectrophotometrically determined by using an HACH DR/2000 spectrophotometer.

The color of the effluent was determined according to the CPPA standard method (15). Before measurement, the pH was adjusted to 7.6 by the addition of 2 M NaOH. The effluent was then centrifuged to remove suspended solids. The clear supernatant was used for the measurement of absorbance at 465 nm against distilled water. Absorbance values can be transformed into color units (CU) according to the equation CU = 500 A_2/A_1, where A_1 is the absorbance of 500-CU platinum-cobalt standard solution (A_{465} = 0.132) and A_2 is the absorbance of the effluent sample.

The AOX and EOX concentrations were determined by using Euroglas Netherlands instrument ECS-2000 according to the manufacturer's recommended procedure.

Chlorophenols and chloroaldehydes were determined by gas-liquid chromatography. Acetylation and extraction were done by the method of Lee et al. (24). Total solids and total suspended solids were estimated by American Public Health Association (APHA) methods 2540-B and 2540-D, respectively (14).

Alkalinity was measured by APHA method 2320-B (14). Toxicity was assessed by a bioassay. It was conducted according to Indian standard method IS-6582-1971 with guppies or zebra fish (21).

The fungus was grown in potato dextrose broth, and levels of the ligninolytic enzymes lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase in the supernatant and in the biomass of the culture were determined. The cell-bound enzymes were determined by disrupting the mycelial pellets with sand in buffer. LiP was determined by the method of Tien and Kirk (41), MnP was determined by the method of Gold and Glenn (20) by oxidation of ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], and laccase was determined by the method of Niku-Paavola et al. (32).

All the analyses were done in triplicate, and average values are reported.

RESULTS AND DISCUSSION

Table 1 shows the analytical results for the effluent sample. Chlorinated phenols and chlorinated aldehydes were also determined. Thirty-seven standards of chlorophenols and chloroaldehydes were run, and 13 types of chlorophenols and 3 types of chloroaldehydes were found in the extraction-stage effluent (Table 2).

Screening of fungi. Of 110 fungal-isolate cultures, 10 showed more than 80% decolorization of extraction-stage effluent at a glucose concentration of 0.1%. Maximum decolorization, on the order of 92%, was obtained in 24 h with isolate R_2, which was identified as *R. oryzae*. Even in the absence of glucose, this culture showed about 78% color removal in 24 h, whereas under similar conditions other cultures removed only 20 to 30% of the color. *R. oryzae* was selected for optimization experiments in order to further improve its efficiency for color removal. To the best of our knowledge, there is no report in the literature concerning color removal from pulp and paper mill effluents by *R. oryzae*.

TABLE 2. Effect of fungal treatment on chlorophenols and chloroaldehydes in extraction-stage effluent

Compound	Concn (mg/liter) in:		Removal (%)[a]
	Untreated effluent	Treated effluent	
2-Chlorophenol	4.98	0	100
4-Chlorophenol	0.1265	0.0076	94 ± 1.4
2,6-Dichlorophenol	0.1130	0	100
5-Chloroguaiacol	9.60	0	100
4-Chlorocatechol	0.2380	0	100
4,6-Dichloroguaiacol	3.79×10^{-3}	0	100
4,5-Dichloroguaiacol	1.16×10^{-3}	0.62×10^{-3}	53 ± 2.3
3,5-Dichlorocatechol	0.0154	0	100
3,4,6-Trichloroguaiacol	3.9×10^{-3}	0.2×10^{-3}	95 ± 1.1
4,5-Dichlorocatechol	0.014	0	100
3,4,5-Trichloroguaiacol	5.385×10^{-3}	0	100
4,5,6-Trichloroguaiacol	0.01466	0.004	72 ± 1.6
Tetrachloroguaiacol	2.6732×10^{-3}	1.12×10^{-3}	58 ± 2.2
2-Chlorosyringaldehyde	0.065	0.033	51 ± 1.6
Trichlorosyringaldehyde	8.9×10^{-3}	0	100
2,6-Dichlorosyringaldehyde	0.029	0	100

[a] Results are reported as means of three measurements ± the standard deviation.

Optimization of process parameters. Process parameters were optimized with the most efficient fungal isolate. The fungus was grown in potato dextrose broth in the form of pellets, thus eliminating the problem of recycling the biomass and making it possible to use a large amount of fungus. The pellets were washed with distilled water before addition to the treatment flasks. The influence of cosubstrate on decolorization was tested individually at a cosubstrate concentration of 1 g/liter. Cosubstrates tested included glucose, sucrose, xylose, carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), lactose, ethyl alcohol, starch, bagasse pith, cheese whey, prehydrolysate liquor, and molasses. Additionally, glucose was tested at concentrations ranging from 0 to 5 g/liter. The concentrations of ammonium chloride, calcium chloride, magnesium sulfate and potassium dihydrogen phosphate in the medium were also optimized. Ammonium chloride was tested at concentrations from 0 to 0.5 g/liter; $MgSO_4$, KH_2PO_4, and $CaCl_2$ were tested at concentrations from 0 to 3.0 g/liter. The effects of pH and temperature on decolorization were studied at pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0 and at temperatures ranging from 25 to 50°C. Effectiveness of cell mass at different incubation times (16 to 96 h) was also tested.

Glucose was found to be the most effective cosubstrate for decolorization. Maximum decolorization on the order of 92%, was obtained within 24 h with addition of glucose. Ninety percent color reduction in 24 h was measured with microcrystalline cellulose and lactose, 89% was measured with sucrose, and 88% was measured with CMC and xylose. Starch and ethyl alcohol showed about 87 and 84% color reduction in 24 h, respectively. With bagasse pith and cheese whey, higher degrees of color reduction, on the order of 85 to 89%, were achieved, whereas with molasses and prehydrolysate liquor the decolorization was found to be suppressed. Eaton et al. compared the suitabilities of three primary sludges and combined sludge with that of cellulose powder for use as a carbon source for *Phanerochaete chrysosporium* cultures (16). The decolorization obtained after 7 days was 82 to 86, 63, and 92% for primary sludge, combined sludge, and cellulose powder, respectively. Ramaswamy observed that addition of 1% bagasse pith as a supplementary carbon source resulted in 80% color reduction in 7 days with *Schizophyllum commune* (38). Experiments by Belsare and Prasad showed that the decolorization efficiencies of *S. commune* with different carbon sources after 7 days of cultivation could be rated in the following order: sucrose (60%), glucose (48%), cellulose (35%), and pulp (20%) (7). Fukuzumi found glucose or ethanol to be most effective for decolorization of waste liquor by *Tinctoporia* (19). Bajpai et al. and Mehna et al. also found glucose to be the most effective decolorization substrate for *Trametes versicolor* (6, 28). Archibald et al. have reported that *T. versicolor* is able to remove color efficiently in the presence of inexpensive sugar refinery or brewery wastes (1).

Color reduction increased with the increase in glucose concentration up to 1 g/liter; beyond that, there was no substantial improvement in color reduction. About 92% color removal was achieved at a glucose concentration of 1 g/liter in 24 h. Even without any glucose, the fungus was able to remove up to 78% of the color in the same period. Studies of color removal by *P. chrysosporium* in a rotating biological contactor showed that decolorization was only slightly affected by the amount of glucose added during the decolorization stage as long as the critical minimum amount of glucose, ~2.0 g/liter, was available (36, 42, 43). Archibald et al. reported a continuous increase in decolorization with an increase in glucose concentration from 1 to 3.5 g/liter (1). In the absence of glucose, no decolorization took place. In contrast, *R. oryzae* showed good decolorization with less glucose, 1 g/liter, or no glucose. Esposito et al. and Lee et al. have reported that the fungus *Lentinus edodes* and the fungus KS-62 also removed 70 to 80% of the color without any glucose (18, 25). However, these fungi took 5 to 7 days to attain maximum decolorization. Neither nitrogen-limiting conditions nor nitrogen supply in excess had any significant effect on decolorization under the conditions tested in the present study. Addition of magnesium sulfate had no significant effect on color removal. Color reduction was found to be maximum at 1.0 g of KH_2PO_4 per liter, 1.5 g of calcium chloride per liter, a pH of 3.0 to 4.0, and a temperature of 30°C, with mycelia harvested after 48 h and an inoculum dose of 0.5 g/liter. Dilution of the effluent from 7,000 to 4,000 PCU increased color removal from 92 to 97%. Beyond that, dilution of the effluent did not cause any increase in color removal.

Decolorization and dechlorination of extraction-stage effluent under optimum conditions. Reduction in COD, AOX, and EOX on the order of 50, 72, and 37%, respectively, was achieved in 24 h. Beyond that, no further reduction in color, COD, AOX, and EOX took place. It was noted that when color reduction reached a certain level, neither additional cosubstrate nor inoculation of the culture filtrate with fresh mycelium resulted in any further reduction in color. This suggests that the effluent contained a very recalcitrant fraction of chromophores that seem to be undegraded under these conditions. Pallerla and Chambers reported 72 to 80% color reduction and 52 to 59% AOX reduction in 24 h with *T. versicolor* (35). With *P. chrysosporium*, AOX and color removal were 40 to 60% and 60 to 80%, respectively, after 1 day of incubation (12, 27). Bergbauer et al. observed maximum color and AOX reduction of 88 and 45%, respectively, in 48 h with *T. versicolor* (8). Initial color concentration of the effluent is a factor influencing the color removal rate. Royer et al. reported a maximum mean decolorization rate of 904 PCU/g of mycelium/day using *T. versicolor* mycelial pellets at an initial color concentration of 3,268 PCU (39). Campbell et al. obtained the color removal rate of 2,000 PCU/day using an oxygen-enriched atmosphere at an elevated temperature of 40°C by employing the MyCoR process with *P. chrysosporium* (11). They also observed a color removal rate of 600 PCU/day using an air atmosphere. Prouty reported an average color removal rate of 1,090 PCU/day using *P. chrysosporium* (37). Pallerla and Chambers obtained a color removal rate of 1,920 PCU/day at an initial color concentration of 2,700 PCU (35). During this study, we observed the highest color removal rate, 6,650 PCU/day, at an initial color concentration of 7,000 PCU.

The porous open-celled structure of polyurethane foam allowed a nondiffusionally limited environment for substrate and product. The decolorization and COD reduction with the immobilized fungus were 95 and 55%, respectively, during the first batch cycle. These values are comparable to those obtained with free mycelium. The immobilized fungus *R. oryzae* retained its decolorization ability for 10 days when treated effluent was replaced daily with fresh effluent. Starting on day 11, however, the decolorization ability of the fungus started to decline. Work with other immobilizing agents to extend the life of the fungus is under way.

Effect of fungal treatment on chlorophenols and chloroaldehydes. After fungal treatment, complete removal of 2-chlorophenol, 2,6-dichlorophenol, 5-chloroguaiacol, 4-chlorocatechol, 4,6-dichloroguaiacol, 3,5-dichlorocatechol, 4,5-dichloroguaiacol, 4,5-dichlorocatechol, 3,4,5-trichloroguaiacol, 4,5,6-trichloroguaiacol, trichlorosyringaldehyde, and 2,6-dichlorosyringaldehyde was achieved (Table 2). The removal of 4-chlorophenol, 4,5-dichloroguaiacol, 3,4,6-trichloroguaiacol, 2-chlorosyringaldehyde, and tetrachloroguaiacol was achieved at levels of 94,

53, 95, 51, and 58%, respectively. The best removals were of the most toxic chlorophenols, trichloroguaiacol, and tetrachloroguaiacol.

Toxicity studies. The initial 50% lethal concentration (96 h) of extraction-stage effluent was in the range of 50 to 55% by volume. After fungal treatment, the effluent was found to be essentially nontoxic. The removal of the highly toxic tri- and tetrachlorophenolic compounds greatly decreased the toxicity.

Enzymatic studies. None of the three ligninolytic enzymes—LiP, MnP, and laccase—were detected in the supernatant of the culture. However, in the biomass, laccase and MnP activities were present and lignin peroxidase was not detected. The maximum MnP activity (0.08 ± 0.005 U/g of mycelium) was observed on the seventh day, while laccase activity was found to be maximum (0.30 ± 0.02 U/g of mycelium) on the sixth day. Studies by several researchers (23, 25, 30) have shown that MnP plays an important role in effluent decolorization in white rot fungi. Laccase has also been implicated in the decolorization of bleaching effluents. Archibald and Roy (2) have reported that laccase plays the primary role in decolorization of bleaching effluent by *T. versicolor*. These researchers demonstrated that *T. versicolor* laccase, in the presence of phenolic substrates, was able to generate Mn(III) chelates similar to those produced by MnP and which were shown by Lackner and coworkers (23) to be responsible for the oxidation of bleaching effluents. Calvo et al. (10) have reported a weak laccase activity in the ascomycete *Paecilomyces variotii* which was found to reduce the color of pulp mill effluent significantly. Christov and Steyn (13) reported decolorization by *Rhizomucor* and suggested that chromophore adsorption on the mycelium was important. So far, to the best of our knowledge the presence of ligninolytic enzymes in zygomycetes has not been shown. There is a need for purification and detailed characterization of these enzymes.

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